Insect Biochemistry and Molecular Biology 105 (2019) 10–24

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Insect venom phospholipases A1 and A2: Roles in the envenoming process and allergy

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ARTICLE INFO

Keywords:
Hymenoptera
Venom phospholipases A1 and A2
Toxic effects
Hypersensitive reactions
Allergy diagnosis

ABSTRACT

Insect venom phospholipases have been identified in nearly all clinically relevant social Hymenoptera, including bees, wasps and ants. Among other biological roles, during the envenoming process these enzymes cause the disruption of cellular membranes and induce hypersensitive reactions, including life threatening anaphylaxis. While phospholipase A2 (PLA2) is a predominant component of bee venoms, phospholipase A1 (PLA1) is highly abundant in wasps and ants. The pronounced prevalence of IgE-mediated reactivity to these allergens in sensitized patients emphasizes their important role as major elicitors of Hymenoptera venom allergy (HVA). PLA1 and -A2 represent valuable marker allergens for differentiation of genuine sensitizations to bee and/or wasp venoms from cross-reactivity. Moreover, in massive attacks, insect venom phospholipases often cause several pathologies that can lead to fatalities. This review summarizes the available data related to structure, model of enzymatic activity and pathophysiological roles during envenoming process of insect venom phospholipases A1 and -A2.

1. Introduction

Insect stings cause a wide range of clinical manifestations including mild to severe toxic reactions, as well as Hymenoptera venom allergy (Golden, 2017; Palma MS, 2013; Vetter, 2018). Massive attacks could trigger different pathologies, such as renal, liver and circulatory failure (Bresolin et al., 2002; Vetter, 2018), severe hemolysis and thrombosis (Lee and Wu, 2004; Yang et al., 2008). In allergic individuals, insect venom could elicit IgE-mediated hypersensitive reactions which can lead to anaphylaxis with potential fatal outcomes (Golden, 2007). Epidemiological studies suggest that 56%–94% of the human population have been stung once in their lifetime and that 9.2%–28.7% shows sensitization to Hymenoptera venoms (Biló et al., 2005). Insect sting is one of the most common elicitors of anaphylaxis cases (27.4%) and anaphylaxis-related fatalities (20%) worldwide (Bilò, 2011; Oropeza et al., 2017).

Clinically relevant insects (Hymenoptera: Aculeata) currently described include several species of bees (Apidae), wasps (Vespidea) and ants (Myrmicidae). The venoms from these insects are complex mixtures of bioactive molecules, small organic compounds (terpenes, biogenic amines), highly abundant linear polypeptides and allergenic proteins (dos Santos-Pinto et al., 2018; Palma, 2013). The low molecular weight compounds and cationic peptides often mediate local toxic, non-fatal reactions (Palma, 2013). Meanwhile, allergens are mainly responsible for hypersensitive reactions and anaphylaxis. To date, 75 venom components from 31 species of social insects have been identified and officially annotated as allergens (www.allergen.org) (Jakob et al., 2017b).

During the last decades, continuous efforts have been made to elucidate the composition of Hymenoptera venoms (Hoffman, 1978; Perez-Riverol et al., 2017; Touchard et al., 2016; Van Vaerenbergh et al., 2014). Venomic analysis of clinically relevant insects have allowed the identification of several proteinaceous toxins, most of them with well characterized enzymatic activity (dos Santos-Pinto et al., 2018). This group of Hymenoptera venom components includes, among others, phospholipases, hyaluronidases, dipeptidyl peptidase IV (DPPIV), proteases and acid phosphatases (dos Santos-Pinto et al., 2018; Hoffman, 2006). Particularly, insect venom phospholipases A1 and -A2 have been extensively described as key toxins during envenoming process and as major hypersensitive reactions and anaphylaxis elicitors (Monsalve et al., 2012; Müller et al., 2012, 1995; Rungsa et al., 2018).

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https://doi.org/10.1016/j.ibmb.2018.12.011
Received 31 August 2018; Received in revised form 10 December 2018; Accepted 18 December 2018
Available online 21 December 2018
0965-1748/

Abbreviations

3-D	three-dimensional structure
bvPLA2	bee venom phospholipase A2
CCDs	cross-reactive carbohydrate determinants
CRD	component-resolved diagnosis
HBV	honeybee venom
HVA	Hymenoptera venom allergy
IgE	immunoglobulin E
PDB	protein databank
PLA1	phospholipase A1
PLA2	phospholipase A2
YJV	yellow jacket venom

Phospholipases are a class of hydrolases that catalyze the hydrolysis of acyl esters and represent one of the earliest enzymatic activity described in nature (Dennis, 2015). Phospholipase A1 and -A2 are toxic and highly allergenic proteins early identified as major components in wasp (Fig. 1A) and honeybee venoms (HBV) (Fig. 1B); respectively (Hoffman, 1978; Hoffman and Shipman, 1976). Phospholipase A1 was also described as a predominant venom allergen in *Solenopsis invicta* (fire ant) (Hoffman, 1987; Hoffman et al., 1988) (Fig. 1A). Insect venom PLA1 (wasps) and PLA2 (bees) have a high diagnostic value and are commonly used as marker allergens during molecular diagnosis of HVA (Jakob et al., 2017a; Monsalve et al., 2012; Müller et al., 2012). Venom PLA1 has been identified in nearly all clinically relevant members of Vespoidea (Jakob et al., 2017b). Meanwhile, PLA2s have been reported as major allergen in several members of *Apis* and *Bombus* genera (Hoffman et al., 2001; Hoffman and Jacobson, 1996; King et al., 1976).

In addition to cause hypersensitive reactions, during the envenoming process insect venom PLA1 and -A2 exert several direct toxic effects (Hou et al., 2016; Prado et al., 2010). Their enzymatic activities mediate the disruption of cellular membranes, pore formation and necrosis (dos Santos-Pinto et al., 2018; Santos et al., 2007). Furthermore, these toxins could cause severe hemolysis (Costa and Palma, 2000; Hou et al., 2016; Watala and Kowalczyk, 1990) and platelet aggregation (PLA1) (Yang et al., 2008), potentially leading to circulatory failure, thrombosis and infarction (Lee and Wu, 2004; Yang et al., 2008). In contrast to hypersensitive reactions which can be triggers by the low amount of venom injected after a single sting, non-allergic, direct toxic effects are fatal only after massive attacks.

Herein, the available data related to the structure, enzymatic activity, pathophysiological effects and overall biological functions during insect-caused envenoming process of Hymenoptera venom PLA1 and PLA2, will be revised. The allergenic activity and their use on novel systems for molecular diagnosis of HVA, will be further addressed. Interestingly, despite their important roles on insect sting-triggered pathologies, to the best of our knowledge, no comprehensive review focusing in Hymenoptera venom phospholipases is currently available.

2. Phospholipases A1 from wasp and ant venoms

2.1. Insect venom phospholipase A1 as enzymes

Phospholipases are classified accordingly to their specific site of action in the substrate. Venom PLA1 from social Hymenoptera belongs to the pancreatic lipase gene family which cleaves ester bonds of 1, 2-diacyl-3-sn glycerophospholipids at the position *sn*-1, releasing the

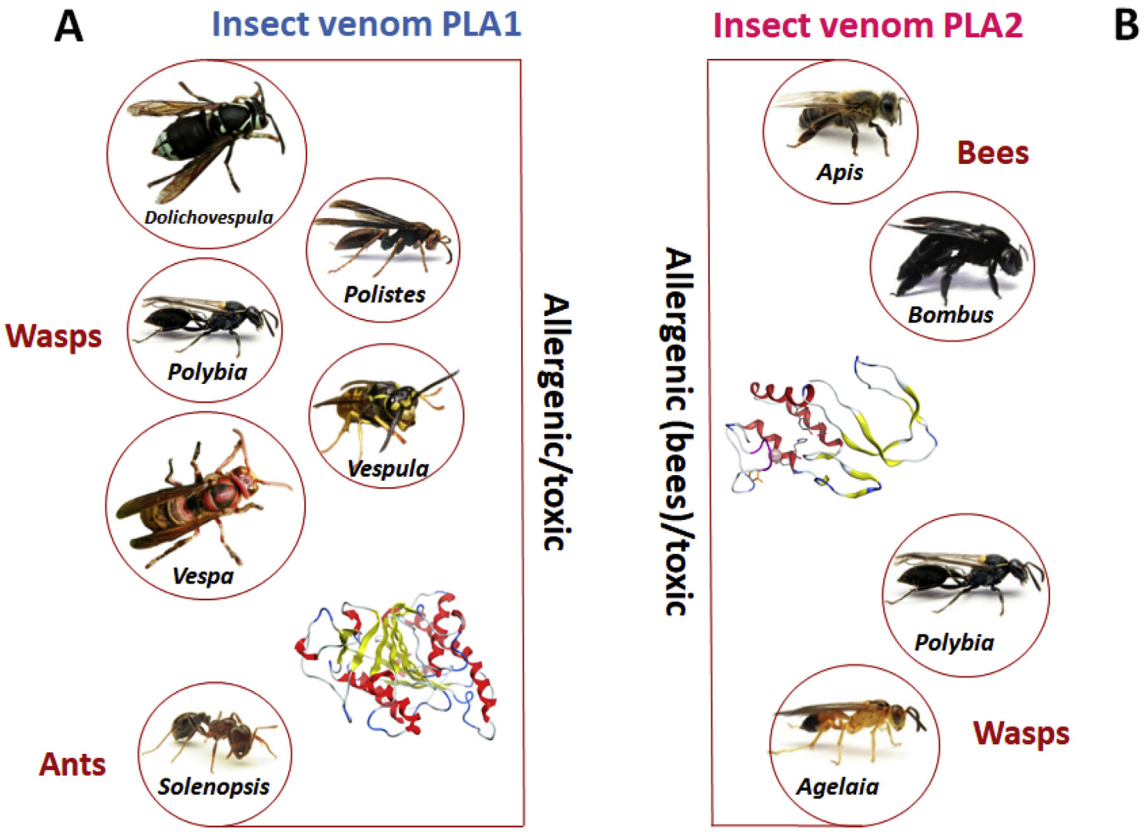

Fig. 1. Clinically relevant Hymenoptera with characterized venom PLA1 **(A)** and/or PLA2 **(B)**. Copyright of the photos from *Apis*, *Bombus*, *Polybia*, *Polistes*, *Agelaia* and *Solenopsis* remains with Prof. Mario Sergio Palma; *Dolichovespula*© photo was modified with permission from Fritz Geller-Grimm/wikipedia.org., under license CC-BY-SA-2.5; photos of *Vespa*© and *Vespula*© were modified with permission from Jelle Devalez/Habropoda, flickr.com.

corresponding lyso compounds (Santos et al., 2007). Typically, insect venom PLA1s are 34 kDa, non-glycosylated proteins that share around 20–30% of homology with the human pancreatic lipase (Hou et al., 2016). Despite, sharing a common substrate, PLA1 and PLA2 clearly differs in their specific site of hydrolysis (Fig. 2A), molecular weight (Fig. 2B) as well as structural characteristics. The primary sequences from wasps and ant venom PLA1 showed no significant levels of identity when compared to apian PLA2 (Fig. 2C). Typically, the tertiary structure of venom PLA1 from different Hymenoptera is composed by 11 parallel/antiparallel β-strands that form a β-sheet in the protein core surrounded by 10 α-helices, which correspond to the classical α/β fold of animal lipases (Hoffman et al., 2005; Hou et al., 2016; Konno et al., 2007; Seismann et al., 2010b). The 3-D structure of these toxins is stabilized by several disulfide-bridges (Perez-Riverol et al., 2016; Santos et al., 2007).

During the last decade, the 3-D models of several insect venom PLA1 have been elucidated using data from sequencing analysis and *in silico* tools (Borodina et al., 2011; Hoffman et al., 2005; Santos et al., 2007; Sukprasert et al., 2013). Furthermore, the crystal structure at 2.5 Å resolution of the PLA1 from *Vespa basalis* (lethal toxin) was recently determined by X-ray crystallography (PDB:4QNN) (Fig. 3A) (Hou et al., 2016). Similar to other members of α/β hydrolase family, the catalytic core of the Hymenoptera venom PLA1s currently described is formed by a Ser-His-Asp triad (Fig. 3B) (Hou et al., 2016; Santos et al., 2007; Sukprasert et al., 2013). Unlike HBV PLA2s, the enzymatic activity of the hymenopteran venom PLA1 is not abolished in the absent of calcium ions, suggesting the lack of a Ca^{2+}-binding site. Vespid PLA1 also contains an amphipathic lid domain which is responsible for the interaction with the hydrocarbon chains of the substrate (Hou et al., 2016). Particularly, α5, α7 and α8 helices as well as the β9 loop from lethal toxin were further identified as important structural factors involved in phospholipid binding and substrate selectivity; respectively (Hou et al., 2016).

Using the crystallographic data from the lethal toxin and the *in silico* modelling of its interaction with the phospholipid PC, a general mechanism of chemical catalysis for insect venom PLA1s was recently proposed (Hou et al., 2016). Overall, the model suggests that initially the short lid domain and β9 loop interact with the phospholipid polar heads. Immediately, the hydrophobic residues in the α5 helix recruit the substrate to the active site which in lethal toxin is formed by Ser137-Asp165-His229 residues (Fig. 3B). Then, the side chains of conserved residues (Phe53, Phe62, Met91, Tyr99, Leu197, Ala167 and Pro169) form hydrophobic channels that stabilize the binding to *sn*-1 and *sn*-2 positions of the acyl chains. Finally, the catalytic triad hydrolyzes the substrate specifically at position *sn*-1 (Hou et al., 2016). The enzymatic activity of hymenopteran venom PLA1 mediates the disruption of phospholipids packing of biological membranes and pore formation, eventually leading to the cell lysis (Santos et al., 2007).

2.2. Phylogenesis of Hymenoptera venom PLA1

Despite their wide diversity regarding toxin arsenal, animal venoms composition also converges in a group of protein families which play crucial roles in the envenoming process (Baumann et al., 2018). Several bioactive components on animal venoms have structurally and functionally homologous in non and/or poor taxonomically related species. Particularly, Hymenoptera venoms evolved as a natural weapon for individual and colony protection as well as for prey capture (Palma MS, 2013). Several social Hymenoptera venom components including allergens such as PLA1 and PLA2 represent bioactive toxins evolutionary selected to cause discomfort and consequently learnt avoidance in colony predators (Schmidt, 2009).

A recent comprehensive phylogenetic analysis on the Aculeata venom allergens showed that allergenic PLA1 from ants and vespid venoms currently described belongs to two different monophyletic clades (Baumann et al., 2018). As authors noted, Formicidae sequences are more closely related to each other than to Vespidae and vice versa, overall suggesting that the PLA1 diversification occurred independently in both families. Remarkably, the study was conducted using only PLA1 sequences from Hymenoptera members. A phylogenetic analysis including PLA1 sequences from other venomous animals such as snake and scorpion as well as social/non-social hymenopteran, also showed that allergenic PLA1 from vespid venoms remain on an isolated monophyletic clade (clade A) (Fig. 4). In contrast, ant PLA1 are located into two different clades. Clade B is constituted by PLA1 from solitary bees, non-social wasps, scorpion, snake and some ants. Meanwhile, clade C is exclusively constituted by ant toxins, including the allergenic PLA1 from *S. invicta*.

The findings obtained using PLA1 hymenopteran sequence clearly indicated that these venom components in social vespid are subjected to an extreme evolutionary conservation (Baumann et al., 2018). Authors found that, as expected from venoms of ancient lineages, most

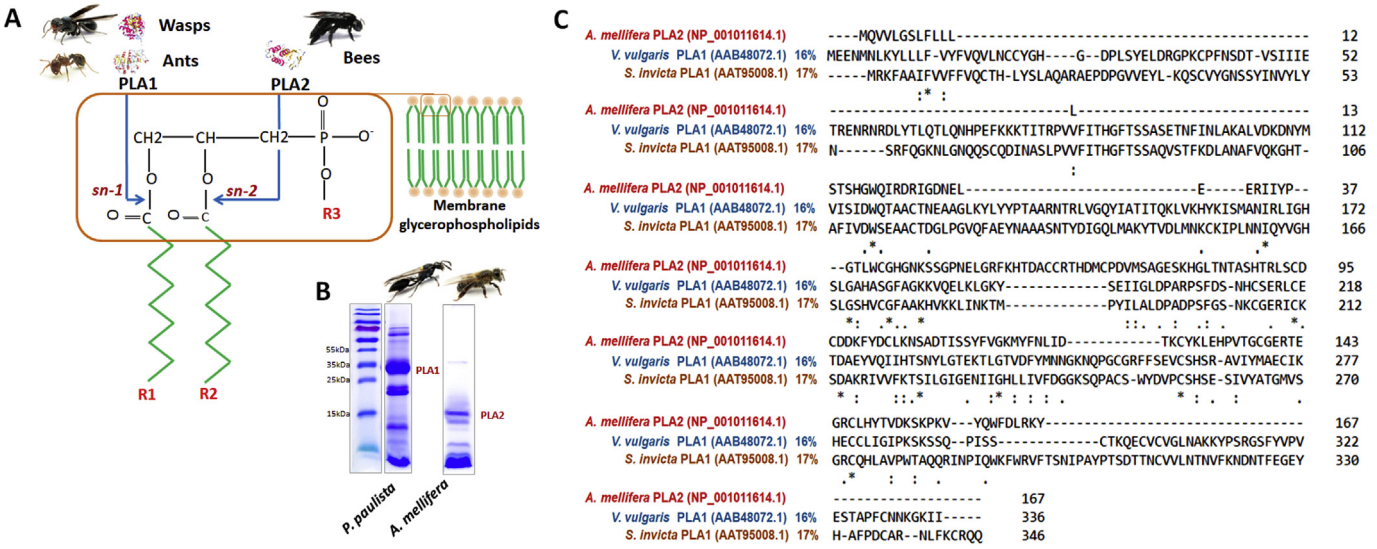

Fig. 2. Differential specificity of the enzymatic activity **(A)**, electrophoretic profile (molecular weight) in SDS-PAGE (12%) **(B)** and primary sequence variations (**C**) of the PLA1 and PLA2 from Hymenoptera venoms. The accession numbers on NCBI databases of the phospholipases and the percentage of identity of wasp and ant venom PLA1s with HBV venom PLA2, are indicated **(C)**. Copyright of the insect's photos remains with Prof. Mario Sergio Palma.

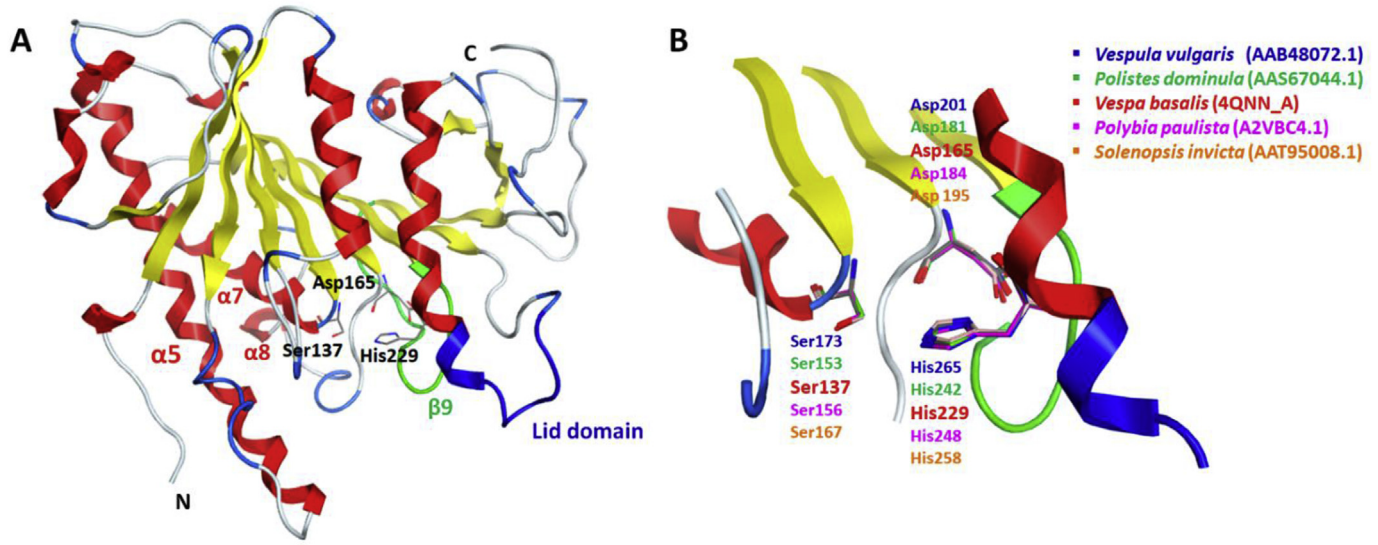

Fig. 3. Ribbon representation of the 3-D structure from *Vespa basalis* wasp venom PLA1 (lethal toxin) **(A)** and the conserved catalytic triad (Ser, Asp, His) from several insect venom PLA1s **(B)**. Structural motifs involved in substrate binding (α5, α7, α8 helices) and selectivity (lid domain -blue- and β9 loop -green-) are highlighted. The ribbon diagrams were prepared with the Molecular Operating Environment (MOE) software (Chemical Computing Group, Canada) and using the deposited PDB of the toxin [PDB: 4QNN (Hou et al., 2016);]. The sequence identifiers on the NCBI databases of the insect venom PLA1s included in the analysis, are showed **(B)**.

hymenopteran PLA1 protein residues evolved under the influence of negative selection. Previous studies also indicate that variations on insect venom toxins have the same pattern of other ancient venomous lineages such as scorpion, in which variations of venom component encoding-genes accumulate episodically under evolutionary pressure from prey and predators (Sunagar and Moran, 2015). In allergenic components, variations concentrated in residues of protein surface while the core and particularly the amino acids from the active site are highly conserved. Toxin alternatives which confer evolutive benefits for the population such as induction of discomfort and evasion of the victim immune system become fixed and are subjected to purifying selection (Baumann et al., 2018; Sunagar and Moran, 2015).

2.3. Phospholipase A1: direct toxic activity in envenoming process

Although, most Hymenoptera venom PLA1 have been studied due to their allergenic activity, these toxins also cause direct toxic effects in the victims (Chen et al., 2008; Ho and Ko, 1988; Santos et al., 2007). The most prominent toxic effects caused by wasp venom PLA1s include cell membrane disruption, pore formation, severe hemolysis and activation of platelet aggregation, potentially leading to *in vivo* thrombosis (Hou et al., 2016; Santos et al., 2007; Yang et al., 2008). Most studies related to toxic effects mediated by these venom proteins have been performed using clinically relevant species from *Vespa* (hornets) (Rungsa et al., 2018; Sukprasert et al., 2013; Yang et al., 2008). Members of this genus are highly aggressive wasps which have been suggested to cause a significant number of accidents with potential fatal outcomes (Liu et al., 2016; Rungsa et al., 2018). Unlike honeybee, fatalities after mass hornet attacks could be caused by as few as 12–100 stings. Massive envenoming often cause kidney and liver failure and affect lungs, pancreas, heart as well as central nervous system (Vetter, 2018).

Early analysis for functional characterization of the lethal toxin (32 kDa) showed that this venom component exhibits a potent hemolytic activity in washed red cells (Ho and Ko, 1988). Remarkably, the levels of hemolysis caused by this hymenopteran venom PLA1 correlated with its lethality in different experimental models. Hemolysis is one of the major toxic effects involved in fatalities caused by *V. basalis* massive attacks (Ho and Ko, 1988). Moreover, intravenous injection of the toxin in rats triggers a sequential blood pressure fall, eventually leading to circulatory failure and the occurrence of hyperkalemia (Ho and Ko, 1988). Further analyses showed that lethal PLA1 from *V. basalis* is also involved in venom-induced local reactions and causes dose-dependent swelling in rats (Ho et al., 1993). The edema formation is mainly mediated by the induction of serotonin release in the victim. Overall, these results suggest that lethal toxin activity plays a key role in the pathophysiological effects experienced by non-allergic individuals after a massive attack.

Magnifin (34 kDa), a PLA1 purified *Vespa magnifica* venom is also a potent toxin that shares 66% and 78% of identity with their counterparts in the venom of *V. basalis* and bald-faced hornet *Dolichovespula maculata* (Dol m 1), respectively. The *V. magnifica* PLA1 induces *in vitro* platelet aggregation (85%) at low concentration (18 nM) (Yang et al., 2008). This biological activity represented a previously undescribed effect for a wasp venom PLA1 and was shown to occur in a dose-dependent manner. Platelet aggregation induced by magnifin could mediates the occurrence of aortic thrombosis, cardiac failure and cerebral infarction, some of the symptoms experienced by the victims after a wasp massive attack (Lee and Wu, 2004). Interestingly, an *in vivo* analysis using magnetic resonance imaging on rabbits previously treated with magnifin also showed the occurrence of thrombosis as well as arteries narrowing. These studies contributed to unravel some of the pathological mechanisms triggered by wasp venom PLA1. Furthermore, these findings could lead to the rational design of PLA1-specific antivenoms to prevent their toxic effects, overall improving the clinical interventions often required by the victims of massive envenoming (Lee and Wu, 2004).

Insect venom PLA1 from other wasp genera as well as from *S. invicta* has been also suggested to be involved in direct toxic activity (dos Santos Pinto et al., 2012; Hoffman et al., 2005; Santos et al., 2007). Similar to *Vespa* venom PLA1s, the native toxin isolated from *P. paulista* (Poly p 1) showed direct hemolytic activity (Santos et al., 2007). The levels of hemolysis were similar to those caused by the cardiotoxin from *Naja naja* venom. A general mechanism of envenoming, proposed after a proteome profiling of the *P. paulista* wasp venom suggests that in addition to its allergenic role, Poly p 1 is involved in pore formation, cell death and tissue damage (dos Santos et al., 2010; Perez-Riverol et al., 2017).

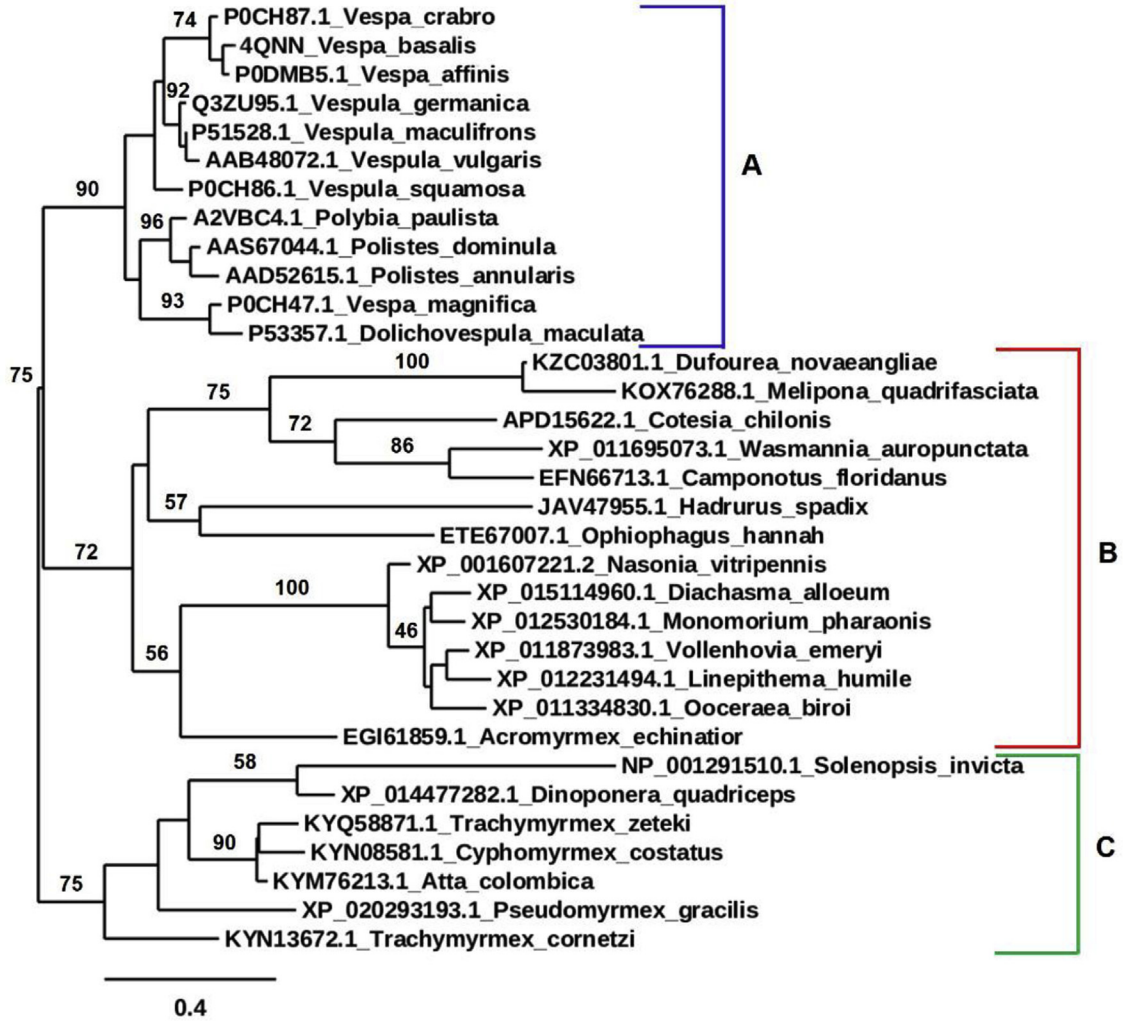

Fig. 4. Phylogenetic tree based on the annotated sequences of phospholipase A1 from social/non-social Hymenoptera as well as scorpion and snake venoms. A, B and C represent closely related groups. Scale bar represents an average of 0.4 substitutions per site. Multiple sequence alignments were performed using the MUSCLE algorithm (v3.8.31) (Edgar, 2004), configured for highest accuracy. The phylogenetic tree was constructed using Phylogeny.fr – PhyML (v3.1/3.0 aLRT) (Dereeper et al., 2008). After alignment, ambiguous regions were removed with Gblocks (v0.91b). Reliability for internal branch was assessed considering statistical tests for branch support – number of bootstraps = 100. The accession numbers (black bold) on NCBI databases of the analyzed PLA1, are provided.

2.4. Insect venom phospholipase A1 as allergens

The immune response elicited by hymenopteran venom allergens such as PLA1 and PLA2 is initiated by the antigen presenting cells (APC) located at the skin, near to the site of the sting. These cells process the allergens and mediate the specific recognition of their linear and conformational epitopes by B and T lymphocytes (Ozdemir et al., 2011). In atopic individuals, naïve T cells activated by APCs differentiate into Th2 cells which, among others, produce the IL-4 and IL-13 cytokines responsible for the class-switching to IgE in B cells. Allergen specific IgE (sIgE) then binds to their specific receptor (FcεRI) in the surface of mast cells and basophils. Subsequent exposure to the sensitizing allergens causes crosslinking of receptor-bound IgE antibodies (Ollert and Blank, 2015), degranulation, release of inflammation mediators such as leukotrienes and histamines which trigger the hypersensitive reactions and in some cases, potentially fatal anaphylaxis (Jutel and Akdis, 2011). Immunotherapy based in preparations of the culprit venom decrease the population of Th2 cells and increase the number of Treg cells, overall resulting on the induction of peripheral tolerance (Ozdemir et al., 2011).

In non-responsive individuals a single sting which typically inject 50–140 μg of venom often results in non-detectable clinical manifestations. Meanwhile, in sensitized patients this amount of venom often causes a wide range of symptoms. These include, generalized cutaneous manifestations such as urticaria, angioedema and pruritus as well as vascular and respiratory disturbances (Golden, 2015). Hypotension, arrhythmias, myocardial infarction, upper or lower airway obstruction, circulatory failure and laryngeal edema are common symptoms during the anaphylaxis and could cause fatalities (Golden et al., 2017). As major hymenopteran allergens, bee PLA2 and wasp/ant PLA1 represent key factors in insect-triggered hypersensitive reactions after their recognition by sIgE in venom-sensitized patients. The high sensitization frequencies described for these allergenic toxins highlight their major role as potent elicitors of HVA.

2.4.1. Wasp allergenic PLA1

Phospholipase A1 are widely distributed among Hymenoptera, being identified as major allergenic proteins in wasp venom from *Vespula*, *Vespa*, *Dolichovespula, Polistes* and *Polybia* genera as well as the fire ant (dos Santos Pinto et al., 2012; Hoffman et al., 2005; Jakob et al., 2017c). In addition to antigen 5, PLA1 represents the most abundant allergenic toxin in most of these insects, typically accounting for up to 6%–14% of the venom dry weight (Jakob et al., 2017c). The sensitization frequencies to PLA1 in patients allergic to yellow jacket venom

(YJV) range from 39% to 79% (Balzer et al., 2014; Ebo et al., 2012; Jakob et al., 2017c; Korošec et al., 2012; Vos et al., 2013). Interestingly, wasps have been identified as the major cause of sensitizations (n = 231) in a cohort of patients (n = 530) with a history of anaphylactic reactions to Hymenoptera (Jakob et al., 2017a). Overall, this epidemiological data highlighted the clinical relevance of insect venom PLA1 as prominent elicitors of HVA.

Currently, a dozen of PLA1s have been officially listed as Hymenoptera venom allergens (www.allergen.org) accounting for up to 27% (12/43) of the wasp venom toxins annotated (Jakob et al., 2017b) (Table 1). Insect venom PLA1 are key tools for differential diagnosis of allergy to HBV and JYV which are the predominant species responsible for insect venom allergy in Western and Central Europe (Ollert and Blank, 2015). As a CCD-free allergen of YJV, with no homologous in apian venoms, PLA1 provide diagnostic benefits for differentiation of serologic double-positivity from the incidence of cross-reactivity (Jakob et al., 2017c; Ollert and Blank, 2015). Combined with antigen 5, the application of PLA1 in routine diagnosis allowed the proper identification of YJV venom sensitizations in up to 92%–98% of the cases (Ebo et al., 2012; Korošec et al., 2012; Müller et al., 2012; Seismann et al., 2010b). Consequently, wasp venom PLA1 represent one of the most widely used wasp allergen in routine diagnosis of insect allergy.

In contrast to double positivity to HBV and YJV, the resolution of genuine sensitizations to Polistinae and/or Vespinae venoms remains challenging (Jakob et al., 2017b). Similarities in homologous allergens such as PLA1 and antigen 5 among these wasps, often prevent differentiation of *Polistes* and *Vespula* allergy. Lately, diagnosis of *Polistes* (European, American and South American paper wasps) sensitizations has gain clinical relevance due to the increased spreading of these insects in United States and Europe (Schiener et al., 2017). Considering that *Polistes* venoms are devoid of CCDs (Blank et al., 2013a; Perez-Riverol et al., 2018b), the protein structural similarities among allergens from YJV and paper wasp venoms represent the unique molecular basis for cross-reactivity and, consequently, the major problem for distinction of sensitization to each insect genera (Caruso et al., 2007; Monsalve et al., 2012; Schiener et al., 2017).

Venom antigen 5 show high levels of sensitization frequencies (84.5%–90%) and are commonly used in molecular diagnosis of wasp allergy. However, wasp and ant venom PLA1 share lower levels of primary sequence identity (30%–77%) (Fig. 5A) as compared to antigen 5 (48%–85%) (Jakob et al., 2017b, 2017a; Perez-Riverol et al., 2018a; Schiener et al., 2017). This is particularly true for European/Southern America paper wasp and YJV, as primary sequence identities for antigen 5 and PLA1 in these insects range from 59% to 62% and (51%–54%); respectively (Perez-Riverol et al., 2018a; Schiener et al., 2017). *In silico* modelling and comparative analysis also revealed that tertiary structure of ant/wasp allergenic PLA1 show higher levels of variations (Fig. 5B), potentially causing peptide-based cross-reactivity at a lesser extends (Jakob et al., 2017b). Thus, despite shows lower sensitization frequencies than antigen 5, PLA1 have emerged as valuable marker for genera-specific diagnosis of allergy in *Polistes* and *Vespula*-sensitized patients. (Monsalve et al., 2012; Perez-Riverol et al., 2018a).

Due to their high clinical relevance and diagnostic value, wasp venom PLA1 have been extensively studied and characterized using proteomic and allergomic approaches (dos Santos et al., 2011; Santos et al., 2007; Sukprasert et al., 2013). Several of them have been produced as heterologous proteins in prokaryotic and eukaryotic cell systems (Jakob et al., 2017a; Seismann et al., 2010b). Particularly, recombinant forms of Ves v 1 and Pol d 1 produced in insect cells are currently available for routine diagnosis of allergy (Jakob et al., 2017b; Monsalve et al., 2012). Meanwhile a recombinant form of the PLA1 from the South American paper wasp *P. paulista* (rPoly p 1) was recently produced in *E. coli* cells (Perez-Riverol et al., 2016). The levels of IgE-mediated reactivity of rPoly p 1 with sera of allergic patients were similar to those obtained with the native toxin and it use prevents the incidence of cross-reactivity during differential diagnosis of HBV and *P. paulista* sensitizations. Thus, rPoly p 1 represent the first venom PLA1 from a clinically relevance Neotropical wasp undergoing evaluation for the development of component-resolved diagnosis of HVA (Perez-Riverol et al., 2018a, 2016).

2.4.2. Fire ant venom PLA1

Fire ant has colonized a broad range of geographical zones of the planet, annually causing significant economic damages and a high number of clinically relevant accidents (Kemp et al., 2000; Steigelman and Freeman, 2013). Similar to bees and wasps, ant venoms specially from *Solenopsis* and *Pachycondyla* genera often trigger direct toxic as well as hypersensitive reactions in the victims (Hoffman, 2010; Lee et al., 2009). In endemic areas, fire ant stings have been informed as the leading cause of allergy cases (42%) among Hymenoptera (Freeman, 1997; Steigelman and Freeman, 2013). In addition to Sol i 2, -3 and -4, hypersensitive reactions to *S. invicta* ant venom are mediated by Sol i 1, the only ant venom PLA1 currently described as Hymenoptera allergen (Hoffman, 2010; Jakob et al., 2017b) (Table 1).

Mature Sol i 1 (34.2 kDa) is a 309 amino acids long protein with three potential N-glycosylation sites on its primary structure (Asn44, Asn72, Asn185) (Fig. 6) (Hoffman et al., 2005). In addition to Dol m 1, from *D. maculata* venom (bald-faced hornet), Sol i 1 represents the only insect venom PLA1 described to carry this type of post-translational modifications (Jakob et al., 2017b). Also, Sol i 1 is the only Hymenoptera venom PLA1 known to have CCD. This structural determinant from Sol i 1 is recognized *in vitro* by the carbohydrate-specific clinically irrelevant IgE in sera from HBV allergic patients, potentially hamper differential the specific diagnosis of fire ant allergy (Hoffman et al., 2005).

Table 1
Insect venom phospholipases officially listed as allergens.

HYMENOPTERA VENOM PHOSPHOLIPASES

Bee venom PLA2

Apis mellifera, A. cerana, A. dorsata
Api m 1, Api c 1, Api d 1

Bombus terrestris, B. pennsylvanicus
Bom t 1, Bom p 1

Ant venom PLA1

Solenopsis invicta
Sol i 1

Wasp venom PLA1

Vespula vulgaris, V. maculifrons, V. squamosa
Ves v 1, Ves m 1, Ves s 1

Polistes dominula, P. gallicus, P. annularis, P. exclamans
Pol d 1, Pol g 1, Pol a 1, Pol e 1

Polybia paulista
Poly p 1

Vespa crabro, V. magnifica, V. mandarina
Vesp c 1, Vesp ma 1, Vesp m 1

Dolichovespula maculata
Dol m 1

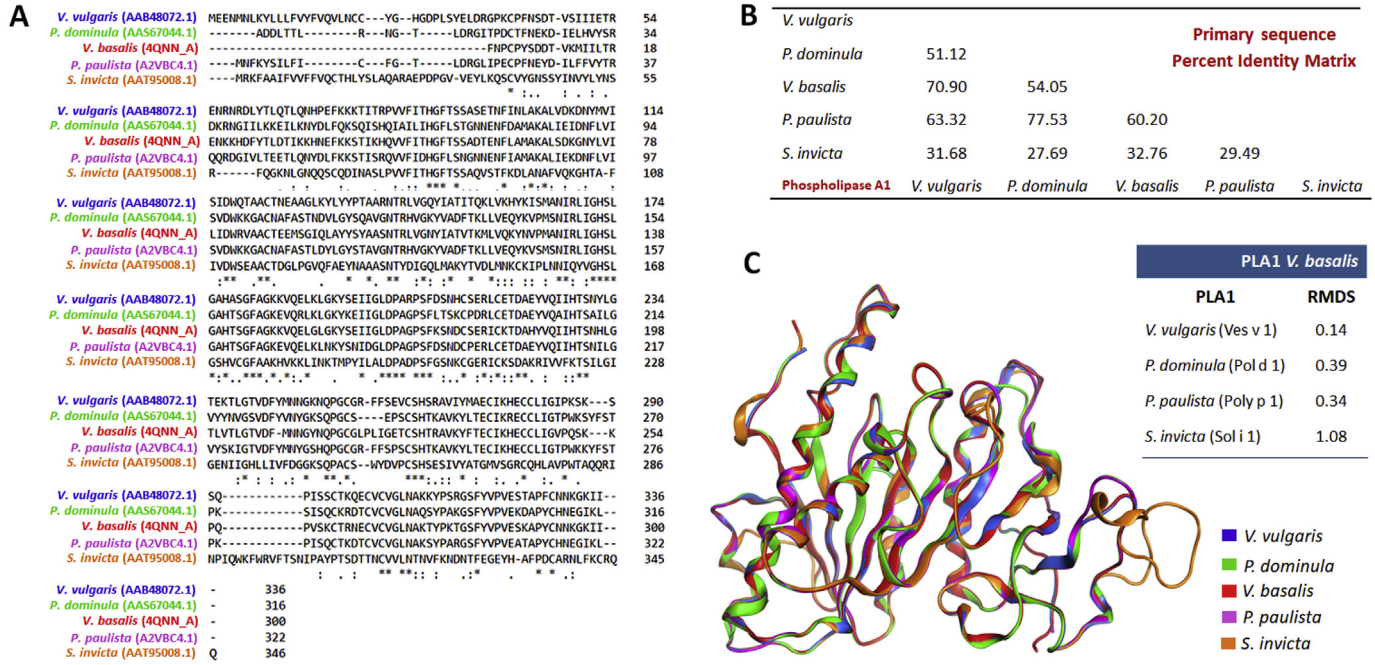

Fig. 5. Venom phospholipase A1 from different clinically relevant Hymenoptera. Multiple alignment (**A**) and level of identity of the protein primary sequences (**B**). The overlay of the PLA1 tertiary structures and the root-mean square deviation (RMSD) of the atomic positions in the 3-D models, are also shown (partially modified with permission from Perez-Riverol et al., 2018a; Molecular Immunology). The 3-D models were generated with MODELLER (Martí-Renom et al., 2000) using the structure of the venom PLA1 from *V. basalis* (PDB ID: 4QNN) (Hou et al., 2016) as general template and the annotated sequences of the insect venom PLA1s [the accession numbers from NCBI databases, are provided in (**A**)]. The models were subjected to energy minimization using YASARA software (Krieger et al., 2009) and then validated using MolProbity (Chen et al., 2010). The ribbon diagrams were prepared with The Molecular Operating Environment (MOE) software (Chemical Computing Group, Canada). The RMSD values were calculated as implemented in MOE software.

Sol i 1 shows different levels of amino acid sequence identity (35%–40%) with their wasp counterparts, with the highest value identified over a fragment of 244 amino acid in PLA1 from Vespula members (36%–38%) and 206 residues from Dol m 1 (40%) (Hoffman et al., 2005). However, the fire ant toxin appears to be more related to *Polistes* venom PLA1 as the longest region of identity (285 amino acid) occurs with Pol d 1. Nonetheless, a recent study suggest that no peptide-based cross-reactivity occurs between Sol i 1 and PLA1 from Polistinae wasps (Perez-Riverol et al., 2018a). The phylogenetic and structural analyses showed above, revealed that Sol i 1 is the most divergent member among the group of currently identified PLA1 from Hymenoptera venoms. As noted, Sol i 1 is located in a clade (clade C) completely isolated from the clade constituted by of wasp allergenic PLA1s (clade A) (Fig. 4). Moreover, in the multiple alignment and the analysis

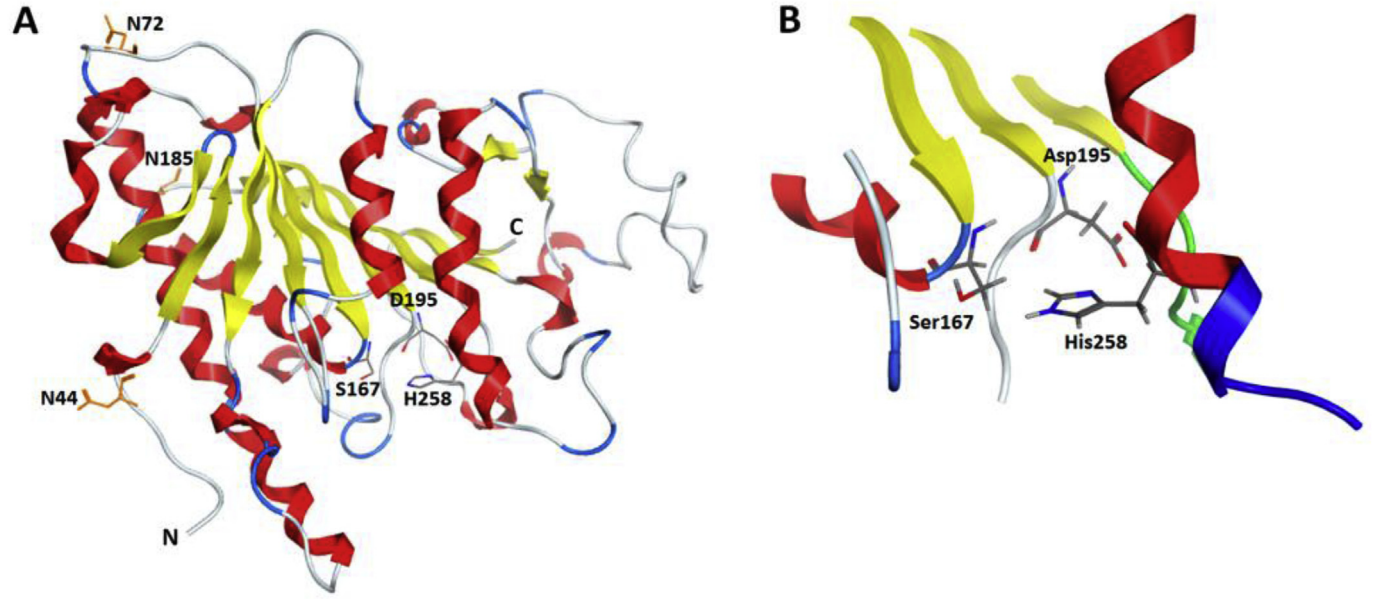

Fig. 6. Ribbon representation of the 3-D structure **(A)** and catalytic core **(B)** of the PLA1 from *Solenopsis invicta*, the unique ant PLA1 currently informed as insect venom allergen. The 3-D model was built as described (Fig. 5 legend), using the PDB:4QNN of the phospholipase A1 from *V. basalis* venom (template) (Hou et al., 2016) and the annotated amino sequence of the fire ant venom PLA1 (GenBank: AAT95008.1).

of 3-D models, the fire ant toxin exhibits the lowest level of sequence identity and the highest value of RMSD, as compared to the wasp venom counterparts (Fig. 5C). Interestingly, despite allergic reactions to *Myrmecia pilosula* ant venom are of great importance in Australia (Brown and Heddle, 2003; Wiese et al., 2007), no venom PLA1 have been identified as major allergen in the venom of this insect.

3. Insect venom phospholipases A2

3.1. Bee venom PLA2: enzymatic activity

Due to the wide diversity of phospholipase A2 currently described in nature, these enzymes have been classified in different groups (GI–GXVI), some of them with several subgroups (Dennis et al., 2011). Bee venom PLA2s (PLA_2-EC 3.1.1.4) belong to the group III (GIII) of small secreted and Ca^{2+}-dependent PLA2s (Dennis et al., 2011). Members of this group are 14–18 kDa proteins with a highly conserved Ca^{2+}-binding motif (XCGXG) and a His-Arg dyad in the active site (DXCCXX**HD**) (Annand et al., 1996; Dennis et al., 2011; Murakami et al., 2015). Bee venom PLA2 requires millimolar concentrations of Ca^{2+} for their catalytic activity. Similar to other members of small secreted PLA2s (sPLA2), the enzyme in HBV showed low specificity for membrane phospholipids (Annand et al., 1996). In contrast to PLA1 (*sn-1*), these enzymes hydrolyze the *sn*-2 ester bond of glycerophohspholipids in biological membranes to release free fatty acids and lysophospholipids (Fig. 2A).

Bee venom PLA2 (bvPLA2) display a highly conserved catalytic network formed by four non-hydrophobic residues (H34, D35, Y87, D64 in *Apis mellifera*) (Zambelli et al., 2017) (Fig. 7). The analysis of the 3-D structure from HBV toxin, led to propose a single-water mechanism for the chemical catalysis mediated by these enzymes (Scott et al., 1990b). The model was suggested based on: (1) the *in silico* simulation of HBV PLA2 interaction with different phosphonate transition analogue states (Scott et al., 1990b; Zambelli et al., 2017) and (2) the mechanism previously proposed for the extensively studied homologous PLA2 in *Naja naja* venom (Scott et al., 1990b; Zambelli et al., 2017). It has been well documented that, despite their significant differences in overall architecture, bee and snake PLA2 have a common mechanism of enzymatic catalysis (Lee and Bae, 2016; Scott et al., 1990a, 1990b).

In the proposed model, the H34 Nδ1 from the active site is initially stabilized by the carboxyl atom of D64. After the substrate binding, H34 Nδ1 atom abstracts a proton from a structurally conserved water molecule triggering the nucleophilic attack of the *sn*-2 position of the substrate. The resulting tetrahedral oxyanion intermediate is stabilized by Ca^{2+} ions provided by the Ca^{2+} binding loop and a backbone amide N−H from Gly10. The Ca^{2+} cofactor is kept in position through the interaction with the carboxyl oxygen atoms of D64, the Ca^{2+} binding loop and two molecules of water. The collapse of this tetrahedral intermediate is followed by the release of the hydrolysis products and the bind of three water molecules to the enzyme active site (Scott et al., 1990b; Zambelli et al., 2017).

3.2. Structure, phylogenesis and toxic role in envenoming process

Hymenoptera venom PLA2 was initially described in *Apis mellifera* using traditional methods for venom fractionation (Hoffman et al., 1976). The bvPLA2 accounts for up to 12% of the HBV and bumble bee venom dry weight (Jakob et al., 2017b). In addition to melittin, apian PLA2 have been described as a prominent toxic factor causing direct cell lysis (Habermann, 1972; Watala and Kowalczyk, 1990). Native PLA2 from *A. mellifera* venom (16–18 kDa) is a highly toxic protein that, unlike most members of sPLA2s (Scott et al., 1990a), harbor an N-glycosylation at Asn 13 residue (Blank et al., 2011; Shipolini et al., 1974a) (Fig. 7A). Apian PLA2 structure contains the insect CCDs which are defined by the presence of an α-1, 3-core fucose (Blank et al., 2011). Insect CCD causes 69%–75% of double positive test results to HBV and (YJV) during allergy diagnosis (Müller et al., 2009; Seismann et al., 2010a).

The protein sequence deduced after the analysis of a venom gland cDNA expression library revealed the presence of a signal peptide, a pro-peptide (15–17 amino acids) and a major peptide of 134 residues (Kuchler et al., 1989). Similar to other members of the small secreted PLA2s group, the primary sequence of the mature toxin showed several cysteine residues involved in the formation of five intramolecular disulfide bridges (Scott et al., 1990b; Shipolini et al., 1974b). The calcium binding motif (^{8}XCGXG^{12}) is sited in one of the protein loops, near to the N-terminal extreme of the molecule. The 3-D structure of the HBV toxin was solved by X-ray crystallography (PDB: 1POC) (Scott et al., 1990a) and showed the typical architecture of sPLA2 composed by three major α-helical segments and a double-stranded antiparallel β sheet and the so called (β wing) (Fig. 7A) (Dennis et al., 2011; Dessen, 2000).

The toxins from bee and the snake are the most extensively studied

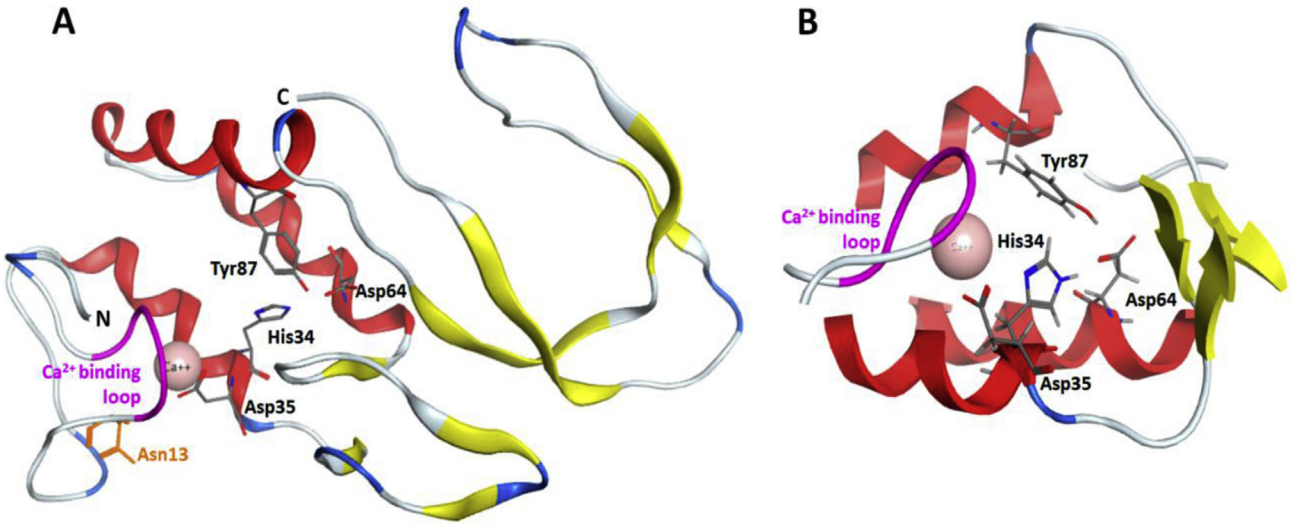

Fig. 7. Ribbon representation of the three-dimensional structure **(A)** and the catalytic network (H34, D35, K87 and D64) **(B)** of *A. mellifera* PLA2 (PDB: 1POC) (Scott et al., 1990a) (NCBI Reference Sequence: NP_001011614.1). The Ca^{2+}-binding site and the N-glycosylated residue (N13) are highlighted in purple and orange; respectively. The ribbon diagrams were prepared with the Molecular Operating Environment (MOE) software (Chemical Computing Group, Canada).

PLA2 from venomous animals. Particularly, apian PLA2 also represent the most well characterized proteinaceous toxin from insect venoms (dos Santos-Pinto et al., 2018; Hoffman et al., 1976). Bee and snake PLA2 show some common structural motifs, including the Ca^{2+}-binding loop and the referred catalytic network (Fig. 7B), which is H48, D49, Y52, D99 in the *Naja naja* venom (Zambelli et al., 2017). Interestingly, despite these structural similarities, bee and snake PLAs significantly differ on their primary sequences. The toxins from Elapidae and Viperidae families belong to groups IA and IIA/IIB of sPLA2, respectively (Zambelli et al., 2017). Meanwhile, as noted, bvPLA2 are members of the group III, which also includes the toxins from lizards and scorpions (Scott et al., 1990a). Venom PLA2 from snake such as several members of *Bothrops* genus also differs from bee PLA2 as they contain enzymatic activity-lacking form of the toxin harboring a D49K mutation (Lys49-subtype of snake phospholipase A2) (Lomonte and Rangel, 2012) which have no bee counterparts currently identified.

The phylogenetic analysis of venom PLA2 using annotated sequences from different venomous animal shows the existence of three isolated clades (Fig. 8). As expected, most snake venom PLA2, are sited on an individual clade (Clade A), different from their bee venom counterparts. Similar to allergenic PLA1, the bee venom toxins also appear in a unique clade (Clade B), suggesting high levels of conservation for these group of venom components. Also as described for PLA1, most variations on allergenic bvPLA2 concentrate in the surface of the protein (Baumann et al., 2018). Interestingly, in addition to social/non-social bee venom PLA2, clade B includes the toxins from lizard (*H. suspectum*) and scorpion (*H. fulpives*) venoms, which, as noted, are also members of the Group III of sPLA2. The toxins from ant venoms grouped in an isolated clade (Clade C) and, as expected, are most closely related to bvPLA2 than their widely studied snake counterparts.

Most severe clinically relevant cases related to the activity of bvPLA2 are associated to the induction of hypersensitive reactions (Golden, 2015). However, this venom component also cause direct toxic effects including local edema formation, induction of cell necrosis and massive liberation of pro-inflammatory mediators (Lee and Bae, 2016; Prado et al., 2010). Apian PLA2 activity also cause direct hemolysis (Habermann, 1972; Watala and Kowalczyk, 1990) and, potentially, pulmonary congestion (Schmidt, 1995). The severity of the symptoms experienced by non-allergic patients often relies on the amount of venom injected. Moderate envenomations (50–200 stings) cause, among other clinical manifestations, abdominal pain, diarrhea, tachycardia combined with hypertension and renal failure (Vetter, 2018). Severe envenoming processes which typically involve 200–500 stings often result in multi-organ failures. Clinical symptoms include blood

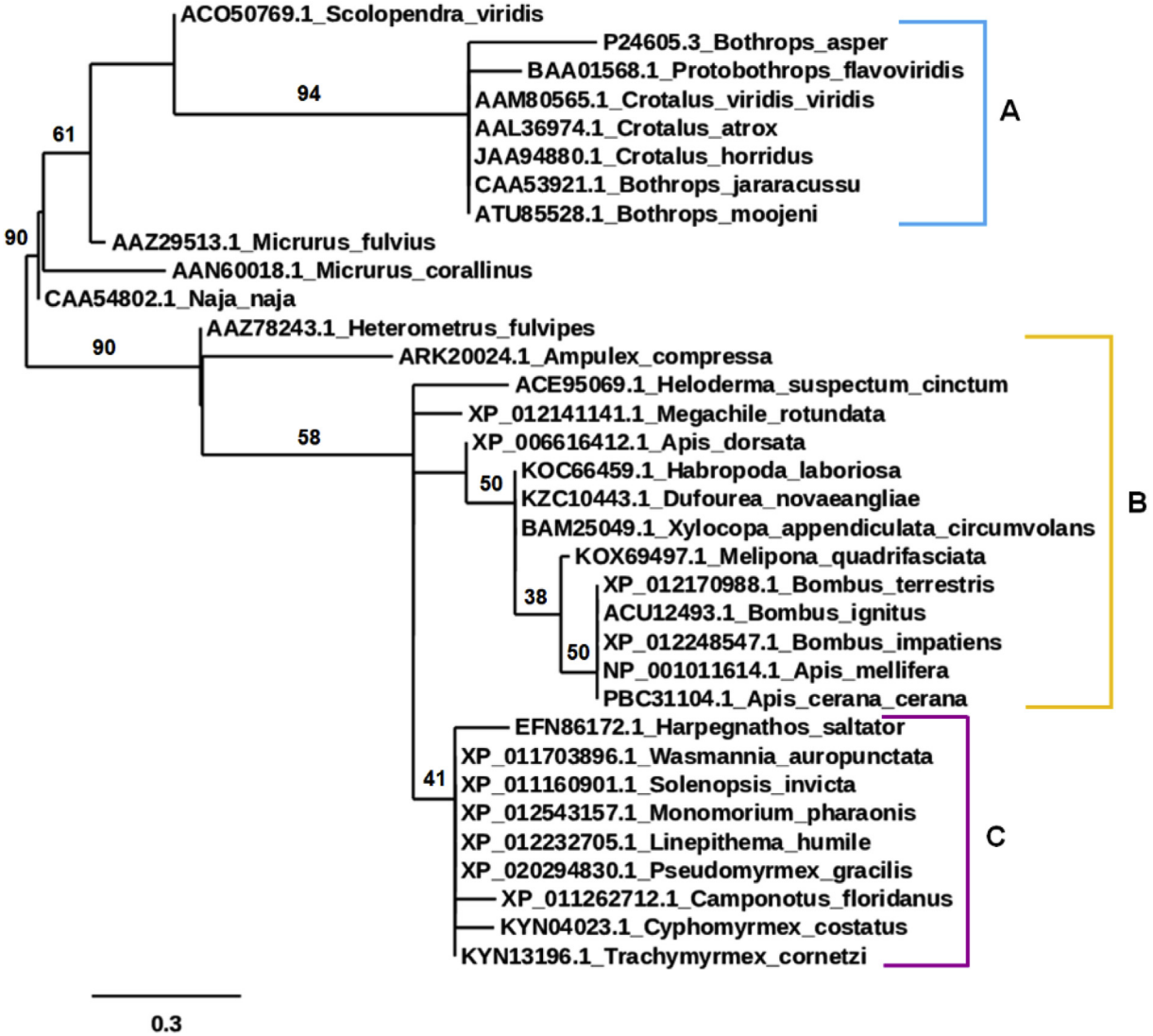

Fig. 8. Phylogenetic tree based on the annotated sequences of phospholipase A2 from social/non-social Hymenoptera, scorpion, snake, lizard and centipede species. A, B and C represent closely related groups. Scale bar represents an average of 0.3 substitutions per site. The accession number (black bold) on NCBI databases of the PLA2, are provided.

hemolysis, rhabdomyolysis, acute renal failure, liver damage, pulmonary congestion and gastrointestinal tract failure (Vetter, 2018; Vetter et al., 1999).

Fatal massive attacks (more than 500 stings) often cause thrombocytopenia, generalized rhabdomyolysis, severe hemolysis, cardiac, liver and renal failure, infarction (Bresolin et al., 2002) and disseminated coagulation (Vetter, 2018). Interestingly, several of these symptoms could be associated to direct toxic activity of bvPLA2. After a massive attack, the toxin has been shown to cause severe myotoxic effects and to impair normal coagulation (Petroianu et al., 2000; Prado et al., 2010). Apian PLA2 activity could increase capillary permeability, potentially leading to blood pressure fall and circulatory failure (Habermann, 2013, 1972). Edema formation, a typical symptom experienced by the victim after a massive envenoming has been also associated to the activity of PLA2 (Cirino et al., 1989).

The direct toxic effects of HBV PLA2 during envenoming are also mediated by a catalytic-independent ligand role (Lee and Bae, 2016). The catalytic-independent activity of bvPLA2 is responsible for nociception effects and neurotoxicity (Lee and Bae, 2016). Subplantar injection of this toxin combined with crotapotin, a peptide which binds PLA2 from South American rattlesnake *Crotalus durissus terrificus* and significantly reduce (60%–70%) the enzymatic activity of bvPLA2, caused the formation of hind paw edema in rats (Landucci et al., 2000). Enzymatically inactive HBV PLA2 is also able to bind the N-type of receptors in the neuronal membranes (Lambeau et al., 1989). Similar to small secreted PLA2 from different snake venoms, the neurotoxicity of HBV PLA2 did not correlated with its enzymatic activity. The high affinity binding of bvPLA2 to N-types of receptor in the nervous system is mediated by structural elements sited at the Ca^{2+}-binding loop and in the protein hydrophobic channel (Nicolas et al., 1997). Unfortunately, the molecular mechanism involved of neurotoxicity of apian PLA2 remains unclear.

3.3. Bee venom phospholipase A2 as relevant allergens

Bee venom is a complex arsenal of allergenic compounds including the melittin peptide as well as several proteins such as hyaluronidase (45 kDa), acid phosphatase (49 kDa), DPPIV (100 kDa), icarapin (55 kDa), vitellogenins (200 kDa) and the predominant PLA2 (Blank et al., 2013b, 2010; Grunwald et al., 2006; Jakob et al., 2017b; King et al., 1976). Particularly, the prevalence of sIgE immureactive to HBV PLA2 in sensitized patients ranges from 58% to 97% (Jakob et al., 2017c; Mittermann et al., 2010; Müller et al., 2009; Selb, 2015). This epidemiology data early indicates PLA2 as a key allergen in bee venom-caused hypersensitive reactions. Moreover, as a major allergen with no homologous in wasp venom which is structurally unrelated to vespid PLA1, apian PLA2 (Api m 1) is used as an ideal marker for bee venom allergy (Blank et al., 2011).

Several studies have informed that bvPLA2 causes liberation of pro-inflammatory mediators in mast cells and basophils (Rigoni et al., 2018). As noted, the cross-linking of receptor-bound IgE antibodies triggered by the allergen recognition on the surface of these cells causes the release of histamine, tryptase, prostaglandins and leukotrienes (Ozdemir et al., 2011). Early analysis reported that HBV PLA2 mediates histamine release from human leukocytes (King et al., 1976; Müller et al., 1995). Interestingly, further analysis showed that Api m 1 is mainly involved in leukotriene C_4 production and up-regulation of mast cells and basophils activation markers such as CD63 (Mustafa et al., 2008). Moreover, the allergen induces the production of the IL-4, the key cytokine involved in Th2 response and class switching to IgE in B-

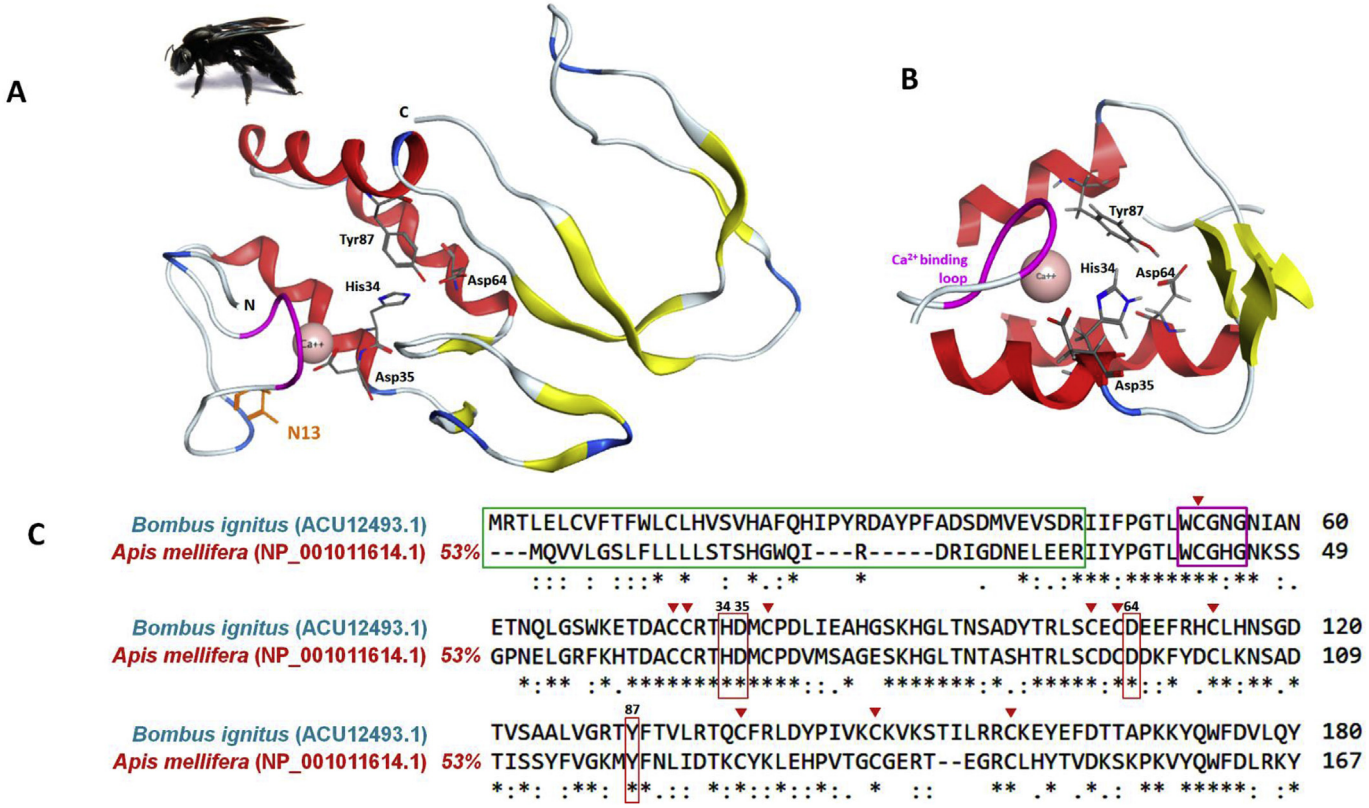

Fig. 9. Phospholipase A2 from *Bombus ignitus.* 3-D model **(A),** catalytic network **(B)** and primary sequence alignment with the HBV PLA2 **(C).** The putative signal/pro-peptide region, the conserved Ca^{2+}-binding site and the catalytic network residues (H34, D35, K87 and D64) of both bvPLA2 are marked with green, purple and red squares; respectively. Red triangles indicate cysteine residues involved in disulfide-bridges formation in *B. ignitus* PLA2. The 3-D model was built as described (Fig. 5 legend), using the PDB: 1POC (Scott et al., 1990a) of the PLA2 from *A. mellifera* venom as template, and the annotated primary sequence of the *B. ignitus* PLA2 (GenBank: ACU12493.1).

cells (Mustafa et al., 2008; Ozdemir et al., 2011). Overall these analyses highlight the important role of Api m 1 on direct activation of the cell types involved in hypersensitive reactions and anaphylaxis caused by HBV.

The recognition of Api m 1 by sera of allergic patients greatly depends on its tertiary structure (Hoffman, 2006). Most of the B and T-cells epitopes identified in the allergen are discontinuous and conformation-dependent (Dhillon et al., 1992; Müller et al., 1995). Consequently, the recombinant forms of the allergen currently used on molecular diagnosis of HVA are produced with a native Api m 1-like fold (Blank et al., 2018; Hofmann et al., 2011; Seismann et al., 2010a; Selb, 2015). As noted, native Api m 1 have CCD which mediates the recognition of the allergen by clinically irrelevant carbohydrate-specific IgE (Blank et al., 2011). For decades, detection of Api m 1 CCD challenge the differentiation of HBV and YJV allergy. Typically, *in vitro* detection of immunoreactive sIgE using crude venom extracts from these insects, showed double positivity in up to 59% of the patients (Müller et al., 2009) hampering the proper identification of the culprit venom. Consequently, several efforts were conducted to produce native-like, CCD-lacking form of this allergen (Eberlein et al., 2012; Hemmer et al., 2001; Seismann et al., 2010a). Currently, CCDs-free variants of Api m 1 produced in *Spodoptera frugiperda* (Sf9) cells are widely used in routine molecular diagnosis for specific identification of HBV allergy (Blank et al., 2011; Jakob et al., 2017a, 2012).

PLA2s have been also identified as major venom allergens in other members of *Apis genus* such as *A. cerana* (Api c 1) as well as bumble bees (Table 1) (Hoffman and Jacobson, 1996; Shen et al., 2010; Xin et al., 2009). Early allergomic analyses performed with several *Bombus* species showed pronounced IgE-mediated reactivity of their venom PLA2 with sera from sensitized patients (Hoffman and Jacobson, 1996). Similar to Api m 1, mature Api c 1 is a 16–18 kDa glycosylated protein of 134 amino acids with high levels of identity with its *A. mellifera* counterpart (95%) (Li et al., 2005). Meanwhile, mature venom PLA2 from *Bombus ignitus* (Bom i 1) (18 kDa) is a 136 amino acids glycoprotein (Fig. 9) that was recently obtained as a recombinant molecule in *Sf9* cells (Xin et al., 2009). The analysis of the primary sequence from Bom i 1 showed the presence of conserved cysteine residues and a bvPLA2-like Ca^{2+}-binding site. Furthermore, Bom i 1 showed different levels of sequence identity with venom PLA2 from other *Bombus* (88%–89%) and honey bee (52%–53%) (Fig. 9C). These levels of protein identities could represent the molecular basis for the diagnostic cross-reactivity previously informed among venoms from members of *Bombus* genera (Hoffman and Jacobson, 1996). Interestingly, on an *in vitro* assay Bomb i 1 induced apoptotic cell death, a previously undescribed role for bvPLA2.

3.4. Wasp venom phospholipase A2

Wasp PLA2 have been described as no-allergenic venom components (Costa and Palma, 2000; De Oliveira and Palma, 1998). These wasp toxins are suggested to cause mild and/or severe toxic reactions depending in the amount of venom injected to the victim. The most prominent pathological effect caused by wasp venom PLA2 is hemolysis. However, due to their lack of allergenicity these insect toxins have been significantly less studied than their bee counterparts. Consequently, limited information is available regarding the molecular and functional characteristic of these wasp toxins. Unlike PLA1, PLA2 are less abundant venom components, typically accounting for 0,1% − 1,1% of the venom dry weight (De Oliveira and Palma, 1998; Diniz-Sousa et al., 2018).

To the best of our knowledge, the first study on wasp venom PLA2 was conducted using the South American paper wasp *Polybia paulista* (De Oliveira and Palma, 1998). Polybitoxins (PbTXs) represented novel types of insect PLA2s and were identified using a classical approach for venom fractionation followed by the evaluation of the PLA2 enzymatic activity of the obtained fractions. PBTxs are highly glycosylated proteins (22%–43% w/w) present as dimeric molecules in the wasp venom. Their catalytic activity depends on low Ca^{2+} ions concentrations suggesting the presence of bvPLA2 − like Ca^{2+}-binding site. Interestingly, PbTXs showed a potent hemolytic activity regardless the presence of any other venom components. Their hemolytic action was higher than of PLA2 from *A. mellifera* (20X), neutral PLA2 from *Naja nigricolis* (17X) and cardiotoxin from *Naja naja* venom (37X).

A similar study performed with the clinically relevant Neotropical wasp *Agelaia pallipes pallipes* (Hymenoptera: Vespidae) (Costa and Palma, 2000) resulted in the identification of another type of wasp PLA2. Agelotoxins (AgTX) (14 kDa) are Ca^{2+}-dependent potent hemolysins also with a high content of carbohydrates on their native structures. Hemolytic activity of the protein was found to be 200 times higher than the PbTXs. Recently, Diniz-Sousa et al. (2018) informed the identification of a snake-like PLA2 (PocTX) in the venom of the *Polybia occidentalis* (Diniz-Sousa et al., 2018). PocTX is a ~14 kDa venom component and similar to some of their snake counterparts harbors the D49K mutation (Lys49-subtype of snake PLA2), consequently lacking enzymatic activity. Interestingly, the sequence of the N-terminal region obtained by Edman degradation showed high similarities with the corresponding region of Lys49-PLA2 from members of *Bothrops* genera such as *Bothrops moojeni* (98.3%) and *B. asper* (94.8%). As authors noticed, several conserved residues in these snake PLA2s such as the Lys49 and Cys 27, 29, 44, 45, 50, 51 and 58 are also present in PocTX.

The identification of a Lys49-subtype PLA2-like protein in wasp venom represents an interesting finding which could improves our understanding of the envenoming process triggered by the insect sting. It has been well documented that snake Lys49 myotoxins causes biological membranes permeabilization by a non-enzymatic mechanism which often lead to severe myonecrosis (Lomonte and Rangel, 2012). Other bioactivities described for this subtype of venom PLA2 includes cytotoxic, myotoxic and pro-inflammatory effects, the induction of cytokine release, degranulation of mast cells as well as edema formation. Interestingly, some of these pathological effects have been informed after a wasp-caused envenoming. Further structural and functional analysis of the toxin will help to explain the symptoms experienced by the victims of massive attacks (Bresolin et al., 2002; Lee and Wu, 2004). Unfortunately, the studies related to wasp venom PLA2s are limited by the low amount of this component in the crude wasp venom (Costa and Palma, 2000; De Oliveira and Palma, 1998) and by the fact that non-allergenic insect venom proteins have been explored at a lesser extent. Moreover, the lack of complete nucleotide/amino acid sequences of these PLA2s in biological databases hampers comparative structural and functional analyses with their bee and snake venom counterparts.

4. *In depth* venomic analyses and novel insect venom phospholipases A1 and -A2

For decades, the analyses of insect venoms composition were based in the use of classical methods. Although this approach allowed the identification of several toxins, technical limitations related to low resolution significantly hampered the detection of novel venom components (dos Santos-Pinto et al., 2018). The low amount of sample extracted from each insect and the heterogeneity in the levels of individual venom compounds, also limited the unraveling of complete insect toxins arsenal. However, recent *-in depth*-venomic studies based in the use of advanced techniques with high dynamic range such as shotgun proteomic has allowed the detection of previously undescribed insect toxin (Van Vaerenbergh et al., 2014), including several putative phospholipase A1 and -A2 (Aili et al., 2016; Peiren et al., 2005; Santos et al., 2017; Van Vaerenbergh et al., 2015).

Using a shotgun proteomics approach (gel-free) combined with a 2-D separation (gel-based) of the *S. invicta* venom, two proteins matching with sequences of insect venom PLA2 previously annotated in insect databases were detected (dos Santos Pinto et al., 2012). As expected, other typical fire ant toxins such as Sol i 1 were also identified. The

detection of a PLA2 in venom of the fire ant represents an interesting finding that can be helpful to understand the pathologies triggered by the insect stings in massive attacks, which are common in accidents involving these social Hymenoptera. However, fire ant venom PLA2 appear to be a non-allergenic toxin as no PLA2-sIgE are detected in patients sensitized to *S. invicta* venom (Hoffman, 2010). A similar proteome screening of *Pachycondyla striata* (Hymenoptera: Formicidae) allowed the identification of a novel putative PLA2 that matches with the amino sequence of vurtoxin, a basic snake PLA2 from *Vipera renardi* (Viperidae). Interestingly, this toxin was reported to block nicotinic acetylcholine receptors in neurons (Vulfius et al., 2014), suggesting that the ant counterpart could also acts as a neurotoxic factor during envenoming. In this study, a PLA1 was also detected as a venom component. Nonetheless, no analysis related to the direct toxic activity and allergenicity of this ant PLA1 is currently available.

A shotgun based venomic analysis of *Neoponera villosa,* an aggressive ant widely distributed in South America, allowed the identification of a bvPLA2-like protein (Pessoa et al., 2016). The detection of this toxin could partially explain the tissue damage and hemolysis mediated by the venom. Similarly, an -in depth-comparative analysis of peptide and protein in venoms from poneroid and formicoid ants, including two other members of *Neoponera*, identified PLA2 as a common component of the toxin arsenal of the species studied (Aili et al., 2016). Moreover, systemic venomics of taxonomically related ant *Paraponera clavata* allowed the detection of a PLA2-like toxin (Aili et al., 2017). Overall, these findings suggest that PLA2 is a key player in the envenoming process causes by ants. Remarkably, unlike bees and similar to wasps, no ant venoms PLA2 have been reported to cause sensitization in the victims, probably due to the lack B and T-cell peptidic epitopes.

In depth proteomics applied to the study of *Bombus* species have also resulted in the detection of either novel or previously described bumble bee venom PLA2. The analysis of the *Bombus terrestris* (bumblebee) venom revealed a wide diversity of compounds including Bom t 1, homologous to Api m 1 in HBV (Van Vaerenbergh et al., 2015). The primary sequences of these allergens share limited levels of identity (53%) and their 3-D structures show low conserved protein surfaces (Hoffman et al., 2001). Consequently, no PLA2-mediated cross-reactivity has been showed to occur among bumble bee and HBV. Furthermore, a similar analysis performed with venoms of five species belonging to *Thoracobombus, a* subgenus of *Bombus* sp, revealed the presence of PLA2 among all the species include in the study (Barkan et al., 2017).

Despite the high dynamic range of the novel techniques used in the referred analyses, which allow the enrichment and detection of low abundant compounds, no PLA1 were detected in the ant venoms from *Paraponera* and *Neroponera* genera (Hymenoptera: Formicidae) as well as in *Bombus* sp. This result showed that the absence of reports related to the presence of PLA1 in bee and some ant species were not related to limitations of the methods used, but to a true lack of the toxins in their corresponding venoms. The application of potent proteomic techniques as well as other *-omic-* approaches will continuously increase our knowledge related to the distribution of PLA1 and PLA2 in insect venoms as well as our understanding of the diversity of clinical symptoms and pathologies they trigger in the victim and preys (Calvete and Lomonte, 2015; Perez-Riverol et al., 2017) (Fig. 10).

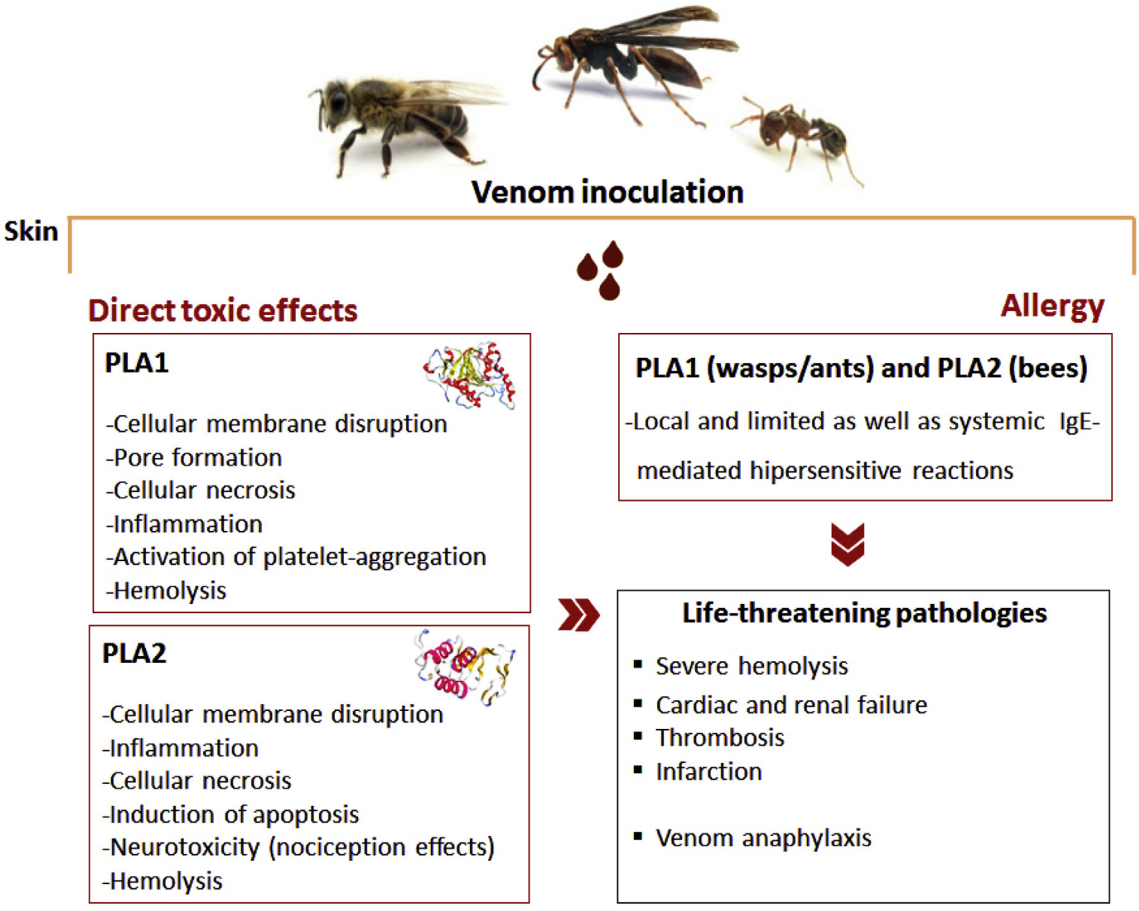

Fig. 10. Summarized toxic effects and types of allergenic reactions caused by the Hymenoptera venom phospholipases and main severe pathologies triggered by these insect toxins in the victim.

5. Concluding remarks

Phospholipases A1 and A2 are among the most predominant proteinaceous compounds in wasps/ant and bee venoms, respectively. Due to the wide range of symptoms mediated by these toxins, they represent key players in the insect sting-triggered envenoming process, particularly on HVA. Direct pathophysiology effects caused by these toxins range from hemolysis, cell membrane disruption, pore formation, necrosis, inflammation, platelet aggregation activation (PLA1) and potentially apoptosis (PLA2). PLA1 are predominant allergens in wasp venoms with no structural homologous identified in bees. Meanwhile PLA2s are major proteinaceous allergenic compounds in HBV and BBV and represent low abundant non-allergenic proteins in wasps. Venom PLA1 and PLA2 from ant/wasps and bees induce high hypersensitive reactions including potentially fatal anaphylaxis and are now routinely used as prominent markers for differential diagnosis of sensitized patients. Further identification and molecular characterization of these molecules in venoms from other clinically relevant insects will certainly improve the CRD of allergy, our understanding of the insect-caused envenoming process and the design of clinical strategies for treatment of the toxic and allergic reactions triggers by the stings.

Ethical statements

This contribution is a review and does not warrants an ethical statement.

Conflicts of interest

The authors declare that there are no personal and professional conflicts of interest.

Acknowledgments

This work was supported by grants from FAPESP - São Paulo Research Foundation (Grant #2016/16212-5), CNPq (Proc. 301656/2013-4) and INCT Program/iii-CNPq/MCT. M.S.P. is a researcher from the National Research Council of Brazil-CNPq. A.P.R. is a Post-Doc Research fellow from FAPESP - São Paulo Research Foundation (Grant #2017/22405-3) at São Paulo State University - UNESP, Rio Claro, Brazil, J.R.A.S.P. is a Post-Doc Research fellow from CNPq (Proc. 150699/2017-4) at São Paulo State University - UNESP, Rio Claro, Brazil and A.M.L. is a researcher from Center for Genetic Engineering and Biotechnology, Department of System Biology, Havana, Cuba.

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